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Effect of Pore Size on the Density of Matrices Made from Collagen Nanofibrils

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***Abstract*—Type I bovine collagen matrices are used for a variety of applications in biomedical engineering and environmental engineering. They are produced from collagen nanofibrils using a multi-step process involving freezing, thermal soaking, freeze-drying and crosslinking of collagen dispersions. The resulting product can be of a variety of geometries and morphologies, but typically maintains the volume of the original dispersion of collagen nanofibrils. There is minimal shrinkage expected during the drying and crosslinking. The density of the final collagen matrix is the mass of the original formulation, minus the liquids, divided by the volume of the formulation; which was verified with measurements. In this research, it was discovered that the matrix density is not a function of pore size or processing specifics, such as thermal soaking time. The density of the collagen matrix was studied as a function of thermal soaking times, which controls the pore size of the matrix. Matrices starting with a formulation by parts (w) of collagen/acid/water equal to 1/5/94 produced a matrix with a density of 13.4 g/l. This is an important discovery in the manufacture of matrices with controllable pore size and morphology to accommodate the specific cell size being grown.**

Keywords—collagen, nanofibrils, crosslinking, porosity, density, lyophilization

I. INTRODUCTION

Collagen is an abundant structural protein that is present as connective tissue in animals. It is insoluble in water, but when recovered as nanofibrils and acidified to a pH value below its isoelectric point of 6.5, can hold and retain many times its own mass in water [1,2,4]. The same is true for the alkaline region although most of the dispersions of collagen nanofibrils (CNF) are produced with the addition of weak organic acids. This is due to the amphoteric nature of the protein and the naturally charged surface. The induced surface charge of CNF is a key physical property that allows the protein to be adapted for a wide variety of practical uses including environmental remediation, purification of biological samples, cell culture, and other engineering and biotechnological applications. Acetic, oxalic, and citric acids have all been used to disperse the nanofibrils [2]. The phase diagram of acetic acid and water was measured with Differential Scanning Calorimetry (DSC) (Fig. 1.) and Nuclear Magnetic Resonance (NMR) [5]. From these data the eutectic for CNF and acetic acid is at -27° C and about 50% (w) acetic acid. The operating acid content is normally 5% (w) for most of the CNF applications.

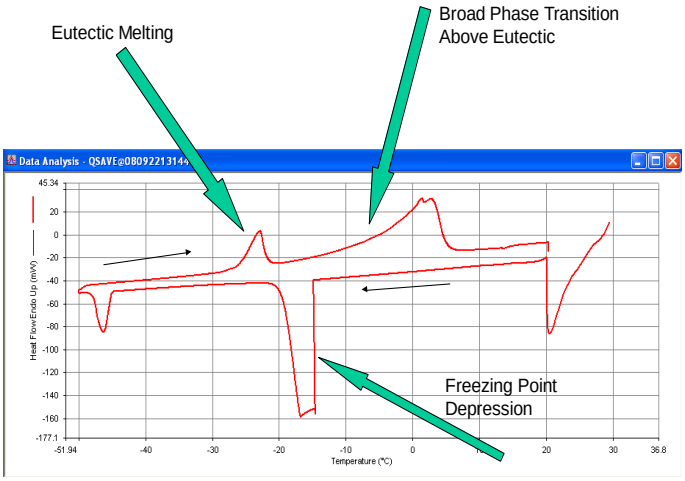

Fig. 1. Phase diagram for collagen dispersions using a DSC.

A. Recovering Nanofibrils from Bovine Corium

Fig. 2. Screening the contents of the ball mill to recover the collagen nanofibrils

The initial processing for collagen matrices is to recover nanofibrils from bovine or porcine corium. Bovine corium, used in this research, is 99% type I collagen with bundles of fibers measuring 5 microns in diameter. This material is obtained from the meat processing industry as a 15% mixture of water and collagen fibers and is designated as raw corium. The milling process has been described in the patent literature [5] and involves extended ball milling in a dilute solution. This

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raw corium is mixed in equal parts by weight with DI water, milled for 7 days and then screened (Fig. 2.). The filtrate is then centrifuged at 5000 rpm and 5° C. The recovered nanofibril paste contains fibrils of about 50 nanometers in diameter and about 300 nanometers in length (Figs. 3. and 4.). The nanofibrils are unraveled from the original collagen fiber, without denaturing, and maintaining the original d-spacing as shown in the AFM micrograph (Fig. 3.).

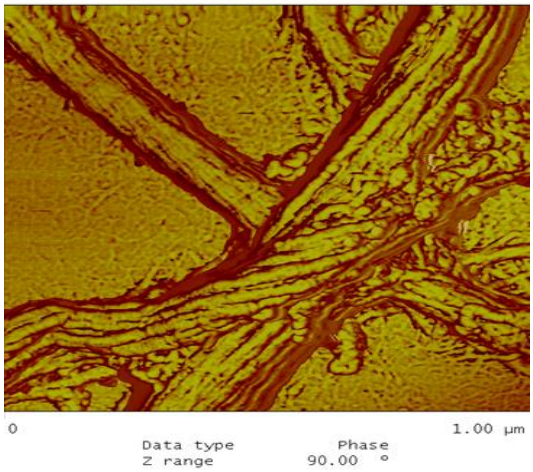

Fig. 3. Atomic Force Microscope (AFM) micrograph of collagen nanofibrils. Note the visible d-spacing.

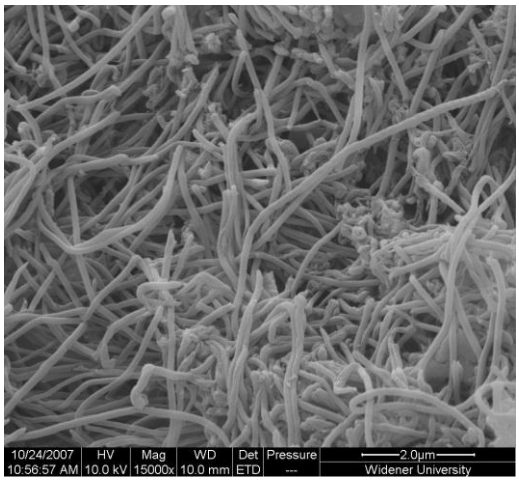

Fig. 4. SEM micrograph of collagen nanofibrils. Average fibril diameter is 50 nm.

In order to produce the viscous 1% collagen dispersion that is the precursor to the collagen matrices, several processing steps are required. The first step is to mill the bovine corium [2]. This was done using a ball mill with milling stones and the mill running for approximately 7 days. The product from the mill was then put in the centrifuge and recovered collagen nanofibrils. In a specific example, this paste was weighed out to 160 grams and was mixed with 64 grams of acetic acid and 1,060 grams of water. These were then mixed to give us the desired composition of the dispersion.

The steps used in this research are summarized as follows:

1. Produce a dispersion with the following constituents: C/A/W = collagen nanofibrils/acid/water = 1/5/94 (parts by weight)
2. Five replicates are made for each experiment. These replicates are studied every ½ hour by moving them from the -15° C freezer to the -80° C freezer. The period in the -15° C freezer creates the pores and the -80° C freezer anchors the pore size and morphology.
3. Freeze dry and crosslink the samples after the thermal soaking step.
4. Analyze the samples using the inverted microscope and the density.

B. Previous Research

Dispersions of collagen have been formed using a starting material of raw fibrillar type I bovine corium using U.S. Pat. No. 6,660,829 [4]. It was found that collagen dispersions can be frozen and then freeze dried, resulting in a product that retains the overall dimensions of the original frozen material. At the same time, over 99% of the volume of the lyophilized product is porous "empty" space and the remaining protein component has a spongy organic aerogel-type structure with controllable pore size, good mechanical properties, and a density as low as one-hundreth of water. It was further found that this solid material can be crosslinked to anchor or memorize its shape, pore size and morphology [3].

Numerous approaches exist for the fabrication of porous materials [9, 10, 11]. Deposition techniques have been used for the fabrication of metal foam. U.S. Pat. No. 4,251,603 and Japanese Patent Application No. 5-6763 describe processes that involve plating a sponge-like resin followed by burning the resin to obtain a metal foam. Freeze-dried collagen dispersions can serve as the sponge-like material to provide a scaffold prior to burning. Such a technique has been described in the patent literature [9]. These processes provide low-density materials having open-cell porosity.

In view of the numerous end uses for porous protein matrices, metal or ceramic materials, there remains a need for structures that are characterized by high porosity and strength. The durability and uniform pore distribution can be made by methods that allow for the adjustment of pore size, porosity, or other characteristics of the final product, depending on the desired end use.

II. MATERIALS AND METHODS – COLLAGEN MATRIX DENSITY

A. Producing Uniform Pore Structure

The density of the collagen matrix is a function of the concentration of collagen in the original dispersion but as discovered in this research not a function of pore size. For this study the original dispersion formulation was held at 1% (w) collagen, 5% (w) acetic acid and 94% (w) distilled water. As described in this section the pore size is controllable and can be made to match the cell which will populate the matrix.

The procedure for the production of a uniform pore structure in the collagen matrix is as follows:

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1. Produce collagen nanofibrils by dilute ball milling as taught in the patent literature [2]. Milling media are 3/8 in cylinders of zirconia.
2. Disperse collagen nanofibrils in a solution of nanofibrils, organic acid, and water. Blend ingredients with a high shear mixer.
3. Freeze the dispersion at a temperature below the eutectic temperature. For mixtures using acetic acid, the eutectic temperature is -27° C and the eutectic composition is about 50% by weight acid. The dispersion phase diagram is shown in Fig. 1.
4. Raise the temperature above the eutectic temperature, but below the freezing point depression line. This is called the soaking temperature and creates liquid and solid regions within the dispersion. The solid region is 100% ice and will eventually comprise the pores upon completion of the process. The liquid phase is at a higher acid content than the original mixture, thus creating a chemical potential and a mobile liquid phase.
5. Hold the dispersion at the temperature in Step 4 for a certain time period. The longer the time and the higher the soaking temperature, the larger the pore size. The time must be long enough to allow the entire sample to reach equilibrium, thus creating a uniform pore size and morphology. This requires about 10 hours for most samples. A sample of the uniform pore size and morphology is shown in Fig. 5.
6. Upon completion of the soaking procedure, the sample should then be refrozen completely to anchor the pore structure. A typical biological freezer operating at approximately -80° C is adequate to refreeze the sample. Material may be stored in the cold freezer at this point before being lyophilized.
7. The lyophilization procedure uses a freeze dryer operating with a -50° C condenser and a chamber pressure of about 0.01 mbar(a). About 2 days are required for primary drying and then another 2 days for secondary drying.
8. Following lyophilization, the sample is typically crosslinked and if necessary sterilized. The crosslinking is accomplished via a dehydrothermal (DHC) process. Such a procedure is accomplished using a vacuum oven operating at full vacuum and 110_o C for 2 days. Sterilization (12 log kill) is achieved by holding the samples at the DHC conditions for 2 weeks [6,7].

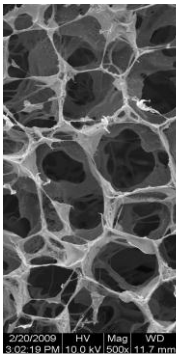

Fig. 5. SEM micrograph of a porous collagen matrix, showing a leafy morphology and a pore size greater than 50 microns

III. DATA: ANALYSIS OF RESULTS

A. *Controlling Pore Size and Measuring Density*

Typically, a phase diagram summarizes the effect of temperature and pressure on a substance in a closed container. For this research the phase diagram of temperature and acetic acid concentration was developed using DSC and NMR [4]. These techniques were described in the patent literature [5]. For the dispersions, the concentration of the acetic acid was at 5% and the samples were frozen at about -80° C. According to the phase diagram (Fig. 1.), at this temperature, the samples are in the solid phase.

At 5% acetic acid, a range of -13 to -16 C temperature allows both a liquid and a solid phase to be present. The water part of the mixture is solidified and forms ice blocks in the mixture, while the collagen part of the mixture (with the acid) remains at the liquid phase. During dry freezing, the ice blocks sublime and leave behind a vacuum forming a porous structure. Going from a low temperature such as -60 C to a higher one such as -13 C permits the water icicles to expand.

Table 1
Initial Work on Pore Size [8]

Soaking Time, h	Soaking Temperature, C	Pore Size, microns
0	-13	5
4	-13	20
10	-13	40-50

Thus the more time the samples are left in the higher temperature, the larger the ice blocks would be. So the longer the samples are left in the higher temperature freezer, the larger the pores would be after placing them in the dry freezer.

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B. *Density Calculations and Results of the Testing*

After the samples were taken out of the vacuum oven, they were all measured with the same caliper and the volume calculated. Next the density was calculated using the measured mass and volume.

The full set of data is summarized in Table 2 for sample size of 63 data points. In Fig. 6. a normal probability plot of the residuals is presented. The residuals were plotted and gave an R2 of 0.97 indicating a strong correlation. In Fig. 7. the actual residuals of the slope in a linear regression are plotted. The data appear to be random and this is further indicated by the normal probability plot which indicate the random nature of the data.

However, the measured densities of all the samples are approximately constant ranging from 5 to 20 kg/m^3, despite their different soaking times.

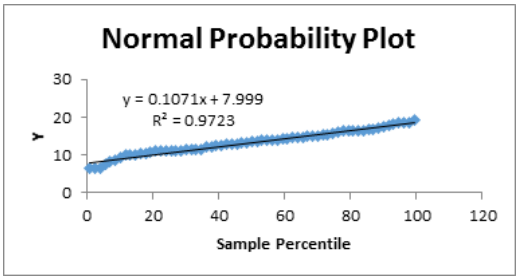

Fig. 6. Normal Probability Plot

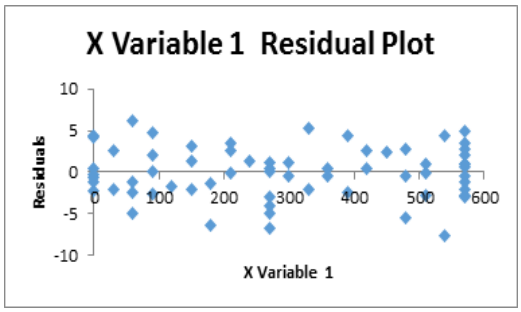

Fig. 7. X Variable 1 (slope) Residual Plot

C. *Goodness of Fit: Hypothesis Testing*

The following hypothesis was tested for the slope of the regression line (Table 2):

H_0: slope = 0 (no relation between the density and the soaking time)

H_1: slope ≠ 0 (there is a relation)

The hypothesis that was set in the beginning of the research is that there is no relation between the porosity of the sample and its density. The outcome of the results obtained indicate that there is no relation between the porosity and the density of the sample. The intercept's confidence interval of 95% is (10.93, 13.66). the t test equals to 17.97. This value does not fall in the confidence interval of the intercept, thus we fail to reject H_0 and we fail to accept H_1. Failing to reject the null hypothesis indicates a relation of 0 value, in other words there is no relation.

Table 2
Regression Statistics for Density (kg/m^3) versus Soaking Time (mins.)

	Intercept	X Variable 1
Coefficients	12.29352	0.003565
Standard Error	0.683955	0.001907
t Stat	17.97416	1.86994
P-value	1.73E-26	0.06614
Lower 95%	10.92675	-0.00024
Upper 95%	13.6603	0.006365

IV. Conclusion

Before the research was started, there had to be an understanding of the effect of temperature on pore size; which is shown through the phase diagram. We wanted to see if the pore size effected the density of the sample which would create time as a variable for density. Through the process of milling the collagen and understanding the phase diagram that was shown above, we were able to use the above equations to get the densities of all 63 samples. By understanding the process at which pore sizes are determined, we were able to set a variable of time in increments of 30 minutes of freeze time. The variable of time, does not affect the density of the sample. The chart, calculations and the data analysis, we have been able to prove that the density of the collagen sample is not effected by the pore size. If a specific pore size is needed, the process to make the sample, does not need to focus on a density.

V. Acknowledgment

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